LAB NEWS

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WINTER FEBRUARY 26, 1971

HELLO!

The State Laboratory Institute has long felt a need to communicate with clinical laboratories in Massachusetts. This newsletter is intended to meet that need. We plan quarterly publication and will distribute copies to all laboratories known to us. We invite your response.

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LABORATORY IMPROVEMENT PROGRAM

Since 1939, the State has operated a program for laboratory improvement. Known as the Laboratory Approval Program until 1970, it then changed to the Laboratory Improvement Program. Approximately 275 laboratories are participants in this voluntary program, which speaks well for its acceptance.

The basic philosophy of the program is that improvement can best be achieved through training and consultation. Since training is expensive, the Laboratory Improvement Program uses proficiency testing to determine direction and priorities.

In 1970, the program expanded its basic areas of microbiology, syphilis serology and immunohematology to include clinical chemistry and hematology. Participation in the new areas has been limited to those who are not in any other external quality control program.

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NEISSERIA MENINGITIDIS

In less than two weeks time (1/25 - 2/4/71) our laboratories received eight cultures of Neisseria meningitidis for confirmation and serotyping. This is the largest number we have received in such a short time period in several years. Nearly all of these cultures were associated with acute illness episodes in young people. Six of the eight come from institutions in the Greater Boston area, one from the Pittsfield area, and another from Holyoke. Laboratories are advised of the importance of prompt sensitivity testing of such

isolates because of the increased number of N. meningitidis strains (especially Group C) now being recovered from clinical specimens that are resistant (> 1 mgm) to the sulfonamide drugs. Also, such sensitivity test findings on the index strain may have considerable bearing on the subsequent management of any case contacts. Cases should be reported to the Massachusetts Division of Communicable Diseases and sub-cultures, if available, should be referred to the Diagnostic Laboratories along with appropriate information both on the patient and the culture. Lately we are seeing mostly Group C's, with an occasional Group B strain. So far, no Group A's (the so-called epidemic strain) have been seen, although last November the first Group A strain from New England in many years was reported from Nashua, New Hampshire. It should also be remembered that occasionally a meningococcal strain may be encountered that cannot be serogrouped with the usually available (A → D) typing sera. In fact, three of the above eight fell in this non-groupable category and were subsequently identified as Slaterus Group Y* strains of N. meningitidis by the Center for Disease Control in Atlanta. The other five included one Group B and four Group C's.

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DRUG SUSCEPTIBILITY TEST FOR MYCOBACTERIA

Although there are no uniform standards for the frequency of drug susceptibility tests on isolations of Mycobacterium sp. from a patient, we recommend that the first isolation from a patient should be subjected to drug susceptibility tests with the primary antituberculosis drugs (INH, SM, PAS), as well as with any other antituberculosis drugs the patient may receive. Following this, monthly specimens are usually submitted. We suggest that if mycobacteria continue to be isolated, these monthly isolations also be subjected to drug susceptibility tests. In this way, emerging resistance to one or more drugs can be detected.

This drug susceptibility test service is available free of charge at various laboratories throughout the State. If you would like a list of these laboratories, call Miss Johnson at 522-3700, ext. 98 (area code 617).

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NIACIN TEST STRIPS FOR M. TUBERCULOSIS

Many laboratories are now finding the use of the commercial niacin test strips to be a most useful aid in the identification of M. tuberculosis. At the same time, we hear from many laboratories that they obtain false negatives when they use the strips.

We called this to the attention of one of the manufacturers who suggested that a detergent residue on the tubes used for the test might be responsible. We performed the strip test with brand new clean tubes, as well as with detergent washed, poorly rinsed tubes.

* cf. Slaterus, K. W., Antonie van Leeuwenhoek. J. Microbiol., Serol. 29, 265-71, 1963 and Oughton, C. J. et al., Appl. Microbiol., 18, 1091, 1969

We then compared these results with results on the same cultures, using the standard Konno Method (Science 124, 985, 1956) for the niacin test. We did get false negatives with the strip test, when we used the "detergent washed-poorly rinsed" tubes, whereas, in every case where the clean new tubes were used, the positives were easily read and they agreed with the standard Konno Method. So, our advice to laboratories having problems with the strip method is to watch their glassware and use known positive and negative controls!

It should also be emphasized that the development of a yellow color in the liquid in the tube indicates a positive test result with the strip method, just as it does with the standard Konno Method. However, as the fluid is absorbed up the strip, the chemicals on the strip will mix and a color will appear at the top of the strip as well. This color change at the top of the strip only indicates that the chemicals have mixed, but it should not be interpreted as a positive result. If you should ever use a strip which does not show a color change at the top, repeat the test with another strip and inform the manufacturer of the problem.

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MEDICARE NOTES

The Social Security Administration has determined that the taking of an EKG tracing (except those performed as part of a routine examination) by an approved independent laboratory will be considered a covered independent laboratory service, provided that certain requirements are met. For further information, contact Miss Helen Healy at 522-3700, Ext. 93.

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DR. GILFILLAN TAKES OVER VIRUS LABORATORY

Robert F. Gilfillan, Ph.D. became the Chief of the Virus Laboratory, a part of the State Laboratory Institute, on January 4, 1971. He replaced Mrs. Joan Daniels who retired after more than 40 years of service.

Dr. Gilfillan was formerly staff virologist in the Pathology and Medical Research Laboratories at St. Margaret's Hospital, Dorchester, Mass. Prior to coming to the Boston area, he was chief of the Virus Diagnostic Laboratory and lecturer in medical virology at the Medical College of South Carolina. A graduate of the University of Tennessee, he is the author or co-author of more than 40 scientific articles.

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PARASITOLOGY TRAINING

The enthusiastic response (50) to the Parasitology Workshop recently announced by the Laboratory Improvement Program was welcomed.

We regret,however, that all could not be accommodated; there were only 16 openings. The course, conducted jointly by the Center for Disease Control (CDC) and the Massachusetts Laboratory Improvement Program, will be given at Simmons College, February 22-26, 1971.

Readers may be interested in the following course offered at the Center for Disease Control, Atlanta, Georgia - May 10-21, 1971:

Laboratory Methods in Medical Parasitology
Part 2 - Blood Parasites

Closing date for applications is March 15, 1971.

There is no charge for courses given by the CDC; however, the applicant will have to bear the cost of travel and living expenses.

All applicants for enrollment in CDC courses must submit a "Training Application" (from PHS O. 319A) through the State Laboratory Institute. These forms are available from:

Mr. Charles Hoye
Administrative Assistant
Diagnostic Laboratories
281 South Street
Jamaica Plain, Mass. 02130

or

Chief, Laboratory Training Section
Laboratory Division
Center for Disease Control
Atlanta, Georgia 30333

or

Chief, CDC Services
Department of Health, Education, and Welfare
Public Health Service
John Fitzgerald Kennedy Federal Building
Boston, Mass. 02203

Other Courses scheduled to be given at the CDC in the near future are listed below:

Course	Dates	Closing Date for Application
Automated Procedures for VD Serology	May 3 - 7	March 26
Basic Laboratory Techniques in Cell Culture	May 21 - 28	March 22
Arthropodborne Encephalitis	June 7 - 11	May 24
Isolation of Salmonellae from Food Products and Animal Feeds	June 14 - 25	April 19

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GENEVA A. DELAND COURSE

The Geneva A. Deland Laboratory Course in Hematology will be offered May 10 - 15, 1971 at Boston City Hospital. This post-graduate course, intended for technologists and physicians, includes intensive training in current methods of laboratory diagnosis and will emphasize cellular morphology and specialized procedures. Contact Herman A. Godwin, M.D., Administrative Director (Tel. 424-4252), Thorndike Memorial Laboratory, 818 Harrison Avenue, Boston, Mass. 02118, for further information.

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PUBLICATIONS

An Ad Hoc Committee of the U.S. Public Health Service has published a report entitled, "Classification of Etiologic Agents on the Basis of Hazard." The Committee's charge was to recommend a system that would help protect the public from infectious agents without restricting bona fide investigations. The report provides a basis for determining the kind of physical containment and level of professional capability required to work safely with hazardous microorganisms. The Laboratory Improvement Program has 200 copies of the report available. To obtain a copy, telephone (617) 522-3700, ext. 90, or write to the Laboratory Improvement Program, address given on the Masthead.

We would like to call your attention to another publication entitled, "Procedures for the Isolation and Identification of Mycobacteria" by Annie L. Vestal. Miss Vestal is the Mycobacteriology Consultant at the Center for Disease Control in Atlanta, Georgia. The book is written in a form that makes it a most useful laboratory tool. Order from the Superintendent of Documents; U.S. Government Printing Office, Washington, D.C. 20402, for $4. The book is Public Health Service Publication No. 1995, published in June, 1969.

Sufficient numbers of a recent monograph on glucose were available for us to append copies to this issue of LAB NEWS. The monograph was prepared by the CDC and is distributed with their permission. We think, and trust you will agree, that it is an excellent, up-to-date resumé of this subject.

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CDC PROFICIENCY TESTING MONOGRAPH

GLUCOSE

QUANTITATIVE DETERMINATION

For a substance with so few distinctive chemical and physical properties as glucose, it is somewhat surprising that its quantification in biologic materials has been even moderately successful. This success derives not from any great selectivity of the older methods, but from the fortuitous patterns of interference in serum. The three main quantitative approaches have been:

(a) Oxido-reduction: The reducing activity of the potential aldehyde group of glucose (that is, the ease with which it is oxidized with mild oxidants) has been successfully applied only because of the relatively high levels of glucose in comparison with the many other reducing materials normally present in serum. In pathologic specimens the interfering substances may increase markedly.

(b) Color reactions: Although there is no distinctive color test for glucose, in recent years a number of rather non-specific reactions have been applied, almost all of them based upon reactions with phenols in strongly acid solution. Several color tests for carbohydrate have been employed, and a mixture of phenol and sulfuric acid is widely used in the very sensitive histochemical detection of carbohydrates in tissue. Sugar group tests abound: pentoses are distinguised by Bial's Orcinol-HCl and other reactions; ketohexoses react fairly selectively with Selivanov's Resorcinol-HCl reagent. The reaction with o-toluidine in acetic acid is fairly selective for aldohexoses, and o-toluidine is a rather successful glucose reagent only because other hexoses are normally present in such low quantities in serum. This fact does not hold true for some pathologic specimens.

(c) Enzymic assays: The potential specificity of enzyme analyses is quite high, and their use will undoubtedly expand as the necessity for greater accuracy in defining serum glucose concentration increases. This discussion will not include enzymic assays, but will be confined to oxidation-reduction and color producing chemical reactions.

I. Metal Ion Reduction

A. Alkaline Copper Reagents

1. The Reaction

Cupric and ferric ions are mild oxidants of substances possessing higher redox potentials (i.e., "reducing" substances). These ions are converted into cuprous and ferrous ions by simple sugars. The

reaction is speeded up with the application of heat. In acid solution, the potential aldehyde groups of most sugars are mildly reducing, but in alkali the mono-saccharides undergo internal Cannizaro reactions which break up the sugars into aldehyde-containing fragments that are more numerous and more reducing than in the original sugar. Although these reactions are not stoichiometric, the rearrangement of glucose and most other hexoses can theoretically produce three two-carbon aldehydes, and the "copper reduction equivalent" of glucose (the number of cupric ions that can be converted into cuprous ions by one molecule of glucose) can approach three. As ordinarily performed, alkaline copper methods yield two or more equivalents of Cu^{+} per glucose molecule, depending on the alkalinity. The heating period is also important, since sufficient time is required to achieve the maximum reduction of copper attainable with any given composition of reagent. With very strong alkali the sugar may be destroyed by oxidation with oxygen.

Effect of acidity of the filtrate: Since alkalinity is so important, any acid contributed by the sample (filtrate) can affect both the rate of the reaction and the final copper equivalent of the glucose present. Consequently, alkalinity can have significant effect on the final results. Older methods used a calibration curve obtained with dilute solutions of pure glucose standards, and the analyst compared the results obtained with the specimen filtrates against the calibration curve. If these filtrates are strongly acid (e.g., contain excess tungstic and sulfuric acids) the actual copper equivalents of the specimens are reduced, leading to underestimation. With supposedly superior procedures in which the glucose standard is treated exactly as the specimen, the acids present in the precipitant are more concentrated in the standard, since the buffering proteins in blood specimens are absent, and the calibration curve proves to be erroneous in the opposite direction. This overestimation is no longer observed with the neutral or mildly alkaline filtrates from metal hydroxides. Treating the calibrating standard in a manner identical to that used for the specimens is a superior procedure in general, but it must be used intelligently.

Reoxidation Errors: In any reaction in hot alkali, atmospheric oxygen becomes a strong oxidant, and unless special means are provided to prevent it, a certain quantity of the cuprous ion formed by reaction with the sugar is reoxidized back into cupric ion. It might be expected that the reoxidized fraction would be proportional to the cuprous ion formed and therefore to the glucose present. However, the reaction is normally heated for

a time interval in excess of that necessary to yield the equivalent quantity of reduced copper--in order to accomodate high glucose concentrations within a linear concentration curve. In effect, oxygen decreases the total cuprous ion by a relatively constant amount in all reaction tubes, producing a "negative" intercept in the apparent standard curve (B in Fig. 1). Curve B, if projected, would indicate that a negative quantity of copper was reduced; actually, curve B may have a somewhat lower slope than indicated, since the copper reoxidized is slightly greater at high glucose concentrations. Note that the minimum determinable glucose concentration is about 25mg% in the example.

The reoxidation effect on glucose analyses by alkaline copper reagents was universal. The old Shaffer-Hartman and Hagedorn-Jensen titrimetric methods had built-in limits to the minimal determinable concentrations at about 40mg% with 1:10 filtrates. Indeed, this explains the early estimates of the "convulsive level" for glucose, and the old definition of the physiologic insulin unit as "producing a decrease in the blood glucose of the rabbit to the convulsive level, or 40mg%". Reoxidation was also seen in the older methods for uric acid, in which the mildly alkaline phosphotungstic acid mixture was heated to 100° for 10 minutes; the errors introduced were quite serious.

Serious analytical errors could be produced in comparing all specimen readings with one selected standard response (curves C or D in Fig. 2). Similar errors can also be made with modern photometers if one-point calibration is used. The use of the old visual (duBoscq) comparator colorimeters masked this phenomenon. Folin himself recognized this source of error, and designed his well-known "sugar tubes" in an effort to minimize reoxidation effects. The original tubes had a constriction of almost capillary dimensions, and when the bulbs were filled with filtrate and reagent, only a very small surface was exposed to air. In time, laboratory personnel complained so vigorously about problems of mixing and cleaning that glassmakers began to taper and widen this constriction until it became useless.

Color reagents: The colorimetric quantification of the cuprous ion produced in alkaline copper reagents has largely revolved around the phosphomolybdic acid complexes. When molybdic or tungstic acids are heated with phosphoric acid or with phosphate ions in strong acid solution, various undefined complexes are formed, all of which are capable of reduction with cuprous ion, but some of which may show other distinctive reactions. Certain phosphotungstate complexes, for example, are used as color reagents

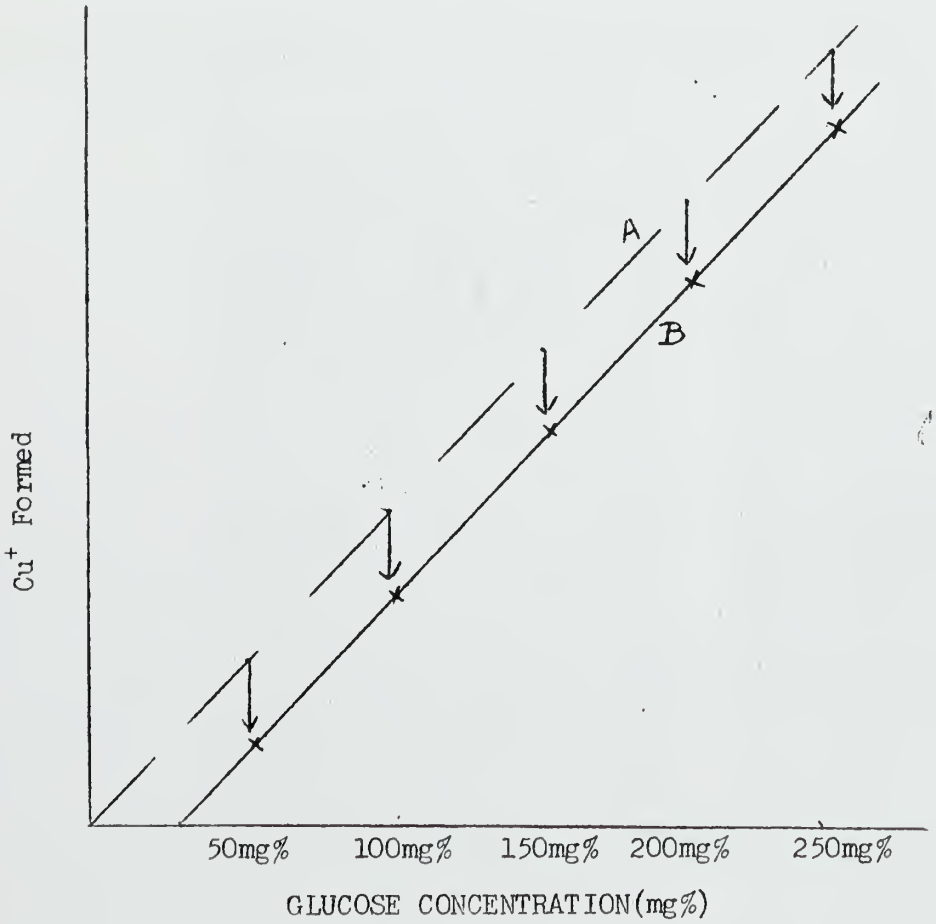

FIG. 1. The theoretical quantities of Cu^{++} reduced to Cu^{+} (Curve A) are diminished by O_2 reoxidation to the points "x" in each reaction tube. Curve B is thus the actual "concentration-response curve" obtained.

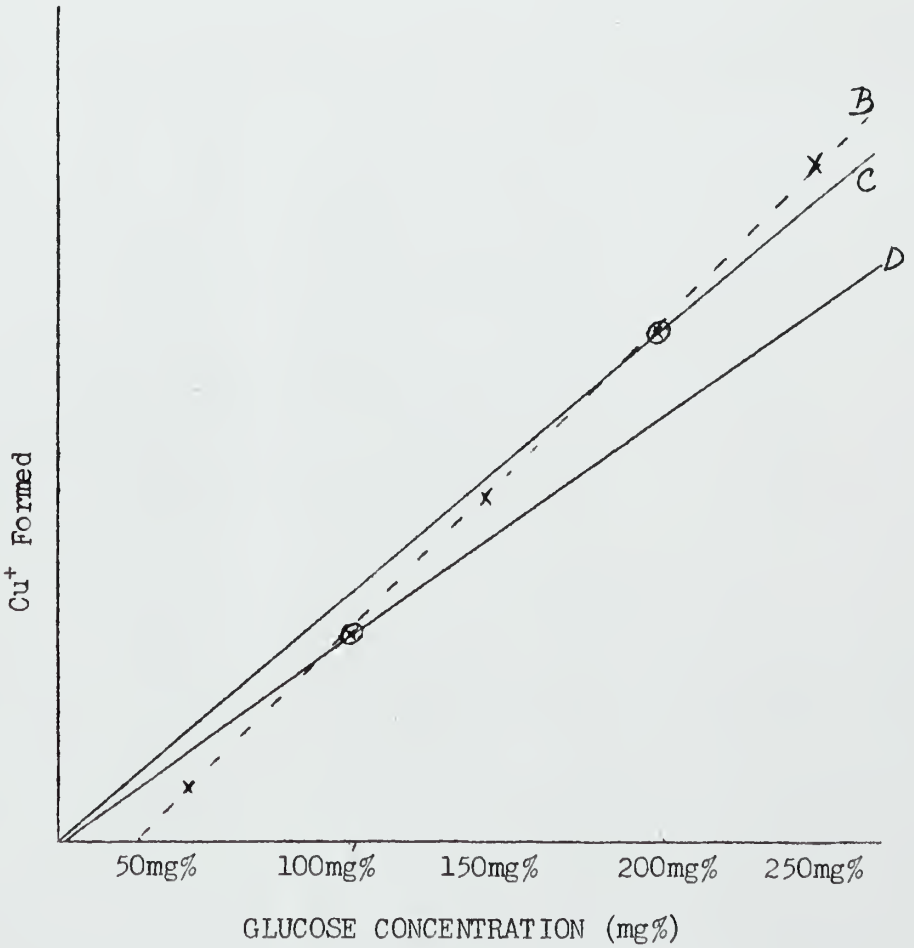

FIG. 2. The effect of computing results from single standard calibrators (actually "two-point" curves, including implied zero). Curve B is the true response curve; C is the curve obtained from the 200mg% standard; D from the 100mg% standard. Note the errors are minimal for samples of concentration close to that of the standard, but rapidly increase in error as the difference increases.

for phenols or are more sensitive to reduction by uric acid, and are generally avoided for glucose analyses. This workhorse reaction is also used in the colorimetric determination of phosphate ion. Reduction produces ill-defined blue products, called "Molybdenum Blue" or "Tungsten Blue", which are not too stable themselves; consequently, special precautions must be taken either to stabilize them or to read colors on a timed schedule. Several complex (and toxic) variants have been used: Benedict's arsenophosphotungstic uric acid reagent and Nelson's arsenophosphomolybdic sugar reagent are examples. Although they are easily reduced in the cold, heating may be used to speed the color reaction, to improve its quantitative proportionality, or even to stabilize it. The reaction may occur in mild alkaline solution (uric acid color assay) or acid, and the reaction pH affects both speed and intensity of color in a rather complex manner.

Color intensities and the use of improper wavelengths: In the conversion of these manual methods from visual colorimetry to photoelectric photometry during the 1940's, another error crept in that has been more difficult to eradicate. The intense colors required for visual comparison led inevitably to the use of concentrated filtrates and large aliquots for most color reactions; these factors were also conducive to nonlinearity of the color densities. Photocell photometry opened a new dimension in the actual measurement of color density as opposed to visual null-point comparison. The nonlinearity of these methods, which then became apparent, was accepted as a fact of life. Instead of diluting out the sample aliquots employed, the intense colors of the Folin and Somogyi reactions were brought down to the readable scale by using wavelengths in the sidebands of the spectral absorption curve. Since biochemical analysts were encountering the physics of color for the first time this was perhaps understandable, but it is inexcusable today.

Accurate determination of concentrations of a colored substance depends vitally upon photometric measurement at its absorption maximum. This is even more crucial with wide bandpass instruments, such as glass-filter photometers or instruments like the Coleman Junior. Many laboratories persist in measuring the intense blue colors of 1:10 glucose filtrates (absorbing beyond 600nm) in the green portion of the spectrum, with continuing problems of nonlinearity. In his book * Henry recommends off-maximum readings for glucose, but his comparisons were made with large aliquots of 1:10 filtrate and extremely intense colors.

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* Henry, R.J., Clinical Chemistry, Principles and Technics, Harper and Row, New York, 649 (1965)

2. The Folin-Wu Assay

The essential deficiencies of this method are determined, not by any of Folin's positive contributions, but by (a) the presence of interfering reducing substances in tungstate filtrates, (b) the uncorrectable differences in acidity between serum filtrates and standards, and (c) the magnitude of the reoxidation problem, which becomes quite serious with dilute filtrates and semimicro methods. Depending upon how the method is calibrated one may obtain a variety of answers--all of them more or less erroneously high. The values given by plasma or serum filtrates are less deviant than for whole blood, and can be reduced by lowering the final tungstate concentration and by adjusting the sulfuric acid. The long feuds between Folin and Benedict and their many modifications of each other's technics did not offer any closer approach, although Benedict once claimed to obtain lower (presumably more correct) values by precipitating whole blood before it was hemolyzed--thus obtaining essentially a "plasma" filtrate upon a measured sample of whole blood!

After 60 years of service-the last 30 of questionable value-it seems time to relegate this method to permanent retirement; this also applies to any oxidation method employing tungstate or other acid filtrates. This can be done quite simply by substituting Somogyi precipitants, alkaline copper, and color reagents for the old ones.

3. Somogyi Methods

Somogyi made two important contributions to the analysis of "true" glucose:

(a) The purified filtrates he obtained from zinc or copper hydroxides remove a large portion of the urate and sulfhydryl interference in serum.

(b) To minimize the reoxidation of Cu^{+} by oxygen, Somogyi saturated his alkaline copper reagent with sodium sulfate. This is so successful in reducing the solubility of O_2 in the reagent that reoxidation is virtually eliminated and even the dilute (1:40 or 1:50) filtrates used with micro methods show almost no "negative" intercept.

Somogyi's approach was quite versatile. His alkaline copper reagents could be used for either titrimetric or colorimetric quantification. For the former, he added iodide-iodate after the glucose reduction of copper and determined the cuprous copper (i.e., glucose) iodometrically; the precision of this method is unsurpassed by colorimetric methods. For the

latter, he employed one or more of Folin's phosphomolybdic acid reagents. Unfortunately, several versions of the "Somogyi" colorimetric assay exist. Somogyi modified his first alkaline copper reagent, primarily by a change in the buffer employed. Nelson "improved" the method by using a new arsenophosphomolybdate color reagent; however, controversy still exists as to whether to reheat the mixture during color development or let it develop in the cold. Any good phosphomolybdic acid reagent will work, but Nelson's modification has become a standard reference method.

"Somogyi" analyses as now performed measure total monosaccharide (preponderantly glucose in normal serum) and a small quantity of other reducing substances. They give some trouble with linearity above 300mg%, the curve often showing increased sensitivity in this area. The method's greatest disadvantage in these days of "quickie" analyses is probably the time and effort consumed in performing its three separate stages.

4. Neocuproine

Neocuproine and certain other substituted phenanthrolines react more or less selectively with cuprous ion, permitting rather sensitive colorimetric assays of microgram quantities of this element in serum. This sensitivity led Brown to use it as a color reagent for microanalyses of glucose, which occurs in concentrations a thousand times those of copper in serum. Unfortunately, unless special precautions are taken to eliminate atmospheric reoxidation of reduced copper in heated alkali, the latter effect proceeds on a relatively large scale. This competition could account in part for some of the odd behavior of this reaction, namely, the high blank color and the negative results we obtained in trying to separate the alkaline reduction from the color reaction.

The reaction was used as a replacement for ferricyanide early in the development of Technicon's SMA system, which required a "forward" color reaction instead of the subtractive ferricyanide method, and this seems to be its only application. Although many users have trouble with it, the SMA continues to use it in lieu of a better one. A similar reaction used for analysis of uric acid in early SMA technology (now generally superseded by older phosphotungstic acid reactions) had inherent errors because the reagent reacts with both uric and ascorbic acids in the dialysate.

So far, we have been unable to pinpoint difficulties some users have with the method. When calibrated with primary standard solutions, the older SMA 12/30 technic yields values with normal sera that may be slightly higher than those from AutoAnalyzer ferricyanide assays (about 4 or 5 mg% higher in recent Proficiency Testing Surveys). With diabetic

patient sera, both assays give results that are sharply increased over glucose oxidase or o-toluidine values. Apparently most problems with the automated method arise from single-point calibration with a high-level serum calibrator and from the continually increasing baseline (drift) which this rather "dirty" reaction produces.

B. Alkaline Ferricyanide Assays

The reduction of ferric ion in alkaline solution has been employed in a number of variants for glucose assay, all of which are subject to the limitations of reductive analysis, and none of which are considered particularly advantageous. An early micromethod, that of Folin and Malmros, depended upon the production of the intense color of "Prussian Blue" from ferric ferricyanide.

The most recent adaptation, Hoffman's alkaline ferricyanide reaction , has seldom been employed manually, but it is the original and current workhorse of the AutoAnalyzer. In alkaline solution and at elevated temperatures, ferricyanide acts as an electron acceptor for the oxidation of glucose; ferricyanide loses color in forming the negligibly colored ferrocyanide ion, and this "subtractive" color reaction is capable of good linearity until the ferricyanide is almost exhausted. Two severe limitations are imposed by any subtractive colorimetric analysis: the initial concentration of ferricyanide in the reagent must be sufficient to span the desired analytical range, and the initial absorptivity of the reagent must be high. The sensitivity in discriminating low and even normal levels of glucose at this high absorbance is therefore quite poor, improving rapidly just as the reagent runs out.

These limitations can trap unsuspecting analysts into rather wide errors. If one forgets to set zero absorbance with a water baseline, for example, it is difficult to judge the usable reagent span, and specimens with elevated glucose concentrations exceeding the reagent capacity can be easily underestimated. This was a particular problem with the early AutoAnalyzers, which were furnished for several years with interference filters of poor quality; stray light from the far red produced decided nonlinearity, masking the point where ferricyanide was exhausted.

The AutoAnalyzer is an extremely flexible system, so much so that it may often be sloppily set up and miserably operated. Consequently, there are an infinite number of "AutoAnalyzer" glucose assays, with a correspondingly wide range of results. In the CDC Proficiency Testing Surveys, AutoAnalyzer ranges for glucose appear to be the widest of any method, and median values run 5-10mg% higher than those with glucose oxidase or o-toluidine. In our own experience, the bias is less than this with pooled serum and with fresh normal specimens and considerably higher with specimens from uncontrolled diabetics at any level.

More specific analyses have been adapted to the AutoAnalyzer and other automation equipment, but certain limitations of the AutoAnalyzer may have slowed their wider adoption. We have found that a direct serum o-toluidine assay works quite well, but the pump tubing delivering the acetic acid reagent must be replaced every day or two. Because the AutoAnalyzer consumes a fairly large amount of reagents, automated glucose oxidase analyses in high volume can become somewhat expensive.

II. Carbohydrate and Sugar Color Reactions

A. Miscellaneous Reactions

There are fads in clinical chemistry as in everything else; ten years ago the anthrone reaction enjoyed a growing popularity as a simplified assay for glucose, possibly because, as a direct serum method employing hot concentrated sulfuric acid, no one expected it to work as well as it did. Almost all carbohydrates react in strong mineral acid solution to yield some form of cyclic aldehyde, most commonly some derivative of furfural. The aldehyde group condenses with a variety of phenolic reagents to yield colors of various quantity and intensity. Polysaccharides, including sugars as well as starches, glycogen, and dextrans, rapidly hydrolyze and react, so that none of these reactions are specific for simple sugars and most of them show only differences of degree in their reactions with specific sugar units: aldoses, ketoses, pentoses, and the like. Since a number of "foreign" sugars or carbohydrates are administered to patients in diagnostic tests or in therapy, color reactions that are broadly non-specific should be avoided, and since we do have a quite satisfactory and more specific assay available in ortho-toluidine, the others can and should be ignored.

B. Ortho-Toluidine Assays

The o-toluidine reaction has become quite popular--deservedly so, since it fulfills the requirements of a simple assay; it is capable of good reproducibility, it yields results that seem to coincide with "true" values for glucose, and it has been difficult to detect the usual faults and limitations one inherently expects from simplified, direct serum color methods. This is fortunate, since it has been widely adopted by the kit-makers specializing in one-reagent direct serum methods. Although for several years our standard o-toluidine analysis was performed upon protein-free filtrates, we have never been able to discern any positive interference with results on straight serum; indeed if any differences exist, the direct results may be barely perceptibly lower, perhaps corresponding to the slight volume displacement of the protein precipitate at 1:20 or lower dilutions.

Chemical mechanisms for the reaction have been postulated, but these seem to be educated guesses. Acetic acid is used as the solvent, and the more anhydrous the reagent the better. The o-toluidine need not be highly pure, and purified reagent may not work as well as the usual technical grade. The solution is customarily stabilized with thiourea.

The reaction is fairly specific for aldohexoses; this specificity essentially limits interference to the occasional occurence of significant levels of galactose in women in late pregnancy and during lactation. Even the abnormal sugars produced in increasing amounts in diabetes do not seem to interfere, since o-toluidine results remain comparable with those of glucose oxidase methods. In fact, if there is any discernable difference between the two sets of results in our hands, glucose oxidase results are occasionally a few milligrams higher, and only hexokinase assays are generally slightly lower. Until contrary evidence is found, therefore, o-toluidine must be considered as much a "true" glucose assay as enzyme methods, which are themselves not absolutely specific.

The extreme sensitivity of the color reaction produces inherent problems in the accurate measurement of semimicro samples: a ratio of sample to total reagent of about 1:100 yields about as much color for the usual analytical range (up to 300mg%) as can be handled by photometry, although the reaction is satisfactorily linear over a wide range of absorbance.

Thus, for a 5ml volume of reagent a sample volume of less than 100 microliters must be delivered, and if the analyst does not use special care and micro sampling equipment of known accuracy and precision, errors of 5% or more may easily be introduced at the outset. Some types of micro samplers, notably the popular air-piston dispensers, show discrepancies of this magnitude between dispensed volumes of aqueous standards and serum samples!

As mentioned above, the o-toluidine method has been automated on the AutoAnalyzer (other types of automation do not usually furnish the high temperatures required). Owing to the need to limit the total quantity of water in the reaction to less than 5% or at most 10%, those methods employing dialysis have had to find ways around this limitation, and these are usually unsatisfactory. A direct assay in which serum is mixed with reagent* has worked well in our hands.

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* Frings et al. CLINICAL CHEMISTRY, 16:282 (1970)